Apold1 deficiency associates with increased arterial thrombosis in vivo

Diaz-Cañestro, Candela ; Bonetti, Nicole R ; Wüst, Patricia ; Nageswaran, Vanasa ; Liberale, Luca ; Beer, Jürg H ; Montecucco, Fabrizio ; Lüscher, Thomas F ; Bohacek, Johannes ; Camici, Giovanni G

Abstract: BACKGROUND Endothelial cells regulate the formation of blood clots thus, genes selectively expressed in these cells could primarily determine thrombus formation. Apold1(apolipoprotein L domain containing 1) is a gene expressed by endothelial cells; whether Apold1 directly contributes to arterial thrombosis has not yet been investigated. Here, we assessed the effect of Apold1 deletion on arterial thrombus formation using an in vivo model of carotid thrombosis induced by photochemical injury. MATERIAL AND METHODS Apold1 knockout (Apold1-/-) mice and wild type (WT) littermates underwent carotid thrombosis induced by photochemical injury and time to occlusion was recorded. Tissue factor (TF) activity as well as activation of mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3 kinase (PI3K)/Akt pathways were analyzed by colorimetric assay and western blotting in both Apold1-/- and WT mice. Finally, platelet reactivity was assessed using light transmission aggregometry. RESULTS After photochemical injury, Apold1$^{-/-}$ mice exhibited shorter time to occlusion as compared to WT mice. Moreover, TF activity was increased in carotid arteries of Apold1$^{-/-}$ when compared to WT mice. Underlying mechanistic markers such as TF mRNA and MAPKs activation were unaffected in Apold1$^{-/-}$ mice. In contrast, phosphorylation of Akt was reduced in Apold1$^{-/-}$ as compared to WT mice. Additionally, Apold1$^{-/-}$ mice displayed increased platelet reactivity to stimulation with collagen compared to WT animals. CONCLUSIONS Deficiency of Apold1 results in a prothrombotic phenotype, accompanied by increased vascular TF activity,decreased PI3K/Akt activation and increased platelet reactivity. These findings suggest Apold1 as an interesting new therapeutic target in the context of arterial thrombosis.

DOI: https://doi.org/10.1111/eci.13191

Apold1 deficiency associates to increased arterial thrombus formation and augmented vascular tissue factor activity and platelet reactivity.

Candela Diaz-Cañestro, PhD,[1*] Nicole R. Bonetti, MD,[1*] Patricia Wüst, MSc,[1] Vanasa Nageswaran, MSc,[1] Luca Liberale, MD,[1,3] Jürg H. Beer, MD,[1,4] Fabrizio Montecucco, [5,6] Thomas F. Lüscher, [1] Johannes Bohacek, PhD,[7,8] Giovanni G. Camici, PhD[1,8,9]

*These authors contributed equally to this work

[1]Center for Molecular Cardiology, University of Zurich, Schlieren, Switzerland; [2]Department of Health Sciences and Technology, Lab of Neuroepigenetics, ETH Zurich, Zurich, Switzerland; [3]First Clinic of Internal Medicine, Department of Internal Medicine, University of Genoa, Genoa, Italy; [4]Department of Internal Medicine, Cantonal Hospital Baden, Baden, Switzerland; [5] IRCCS Ospedale Policlinico San Martino Genoa – Italian Cardiovascular Network, 10 Largo Benzi, 16132, Genoa, Italy. [6] First Clinic of Internal Medicine, Department of Internal Medicine, and Centre of Excellence for Biomedical Research (CEBR), University of Genoa, 6 viale Benedetto XV, 16132 Genoa, Italy; [7] Department of Health Sciences and Technology, Lab of Molecular and Behavioral Neuroscience, ETH Zurich, Zurich, Switzerland; [8]Department of Cardiology, University Heart Center, University Hospital Zurich, Zurich, Switzerland; [9] Department of Research and Education, University Hospital Zurich, Zurich, Switzerland

Corresponding Author: Giovanni G. Camici, PhD, Center for Molecular Cardiology, University of Zurich, Wagistrasse 12, CH-8952 Schlieren, Switzerland.

Email: giovanni.camici@uzh.ch, phone: +41 44 635 64 68, fax: +41 44 635 68 27

Word Count: 4264

Key words: *Apold1*, tissue factor, platelets, arterial thrombosis

Abstract

Background: Endothelial cells and platelets play a fundamental role in regulating the formation of blood clots thus, genes selectively expressed in these cells could primarily determine thrombus formation. *Apold1*(apolipoprotein L domain containing 1) is a gene whose expression is confined to endothelial cells. However, whether *Apold1* directly contributes to arterial thrombosis has not yet been investigated. Therefore, the purpose of this study was to assess the effect of *Apold1* deletion on arterial thrombus formation using an *in vivo* model of carotid artery thrombosis induced by photochemical injury.

Material and methods: *Apold1* knockout (*Apold1*$^{-/-}$) mice and wild type (WT) littermates underwent carotid thrombosis induced by endothelial specific photochemical injury. Time to occlusion was measured in the right carotid artery of both groups. In addition, tissue factor (TF) activity was assessed in the left carotid artery and plasma. Activation of mitogen-activated protein kinases (MAPKs) and phosphatidyl-inositol-3 kinase (PI3K)/Akt pathways were analyzed by Western blotting in aortas of *Apold1*$^{-/-}$ and WT mice. Finally, platelet reactivity in *Apold1*$^{-/-}$ and WT mice was assessed using light transmission aggregometry.**Results:** After photochemical injury, *Apold1*$^{-/-}$ mice exhibited shorter time to occlusion as compared to WT mice. Moreover, TF activity was increased in carotid arteries of *Apold1*$^{-/-}$ when compared to WT mice. Underlying mechanistic markers such as TF mRNA and MAPKs activation were unaffected in *Apold1*$^{-/-}$ *mice*. In contrast, phosphorylation of Akt was reduced in *Apold1*$^{-/-}$ as compared to WT mice. Additionally, *Apold1*$^{-/-}$ mice displayed increased platelet reactivity to stimulation with collagen compared to WT animals.

Conclusions: Deficiency of *Apold1* results in a prothrombotic phenotype, accompanied by increased vascular TF activity,decreased PI3K/Akt activation and increased platelet reactivity. These findings suggest *Apold1* as an interesting new therapeutic target in the context of arterial thrombosis.

Introduction

The formation of blood clots obstructing circulation in vital organs such as the heart and brain represents a major cause of death worldwide.[1] Specifically, arterial thrombosis triggered by endothelial erosion or plaque rupture, referred to as atherothrombosis, is a key mediator of myocardial infarction and ischemic stroke.[1] The pathophysiology of atherothrombosis involves elements pertaining to the atherosclerotic process in itself as well as its interaction with thrombotic complications.[2] Indeed, the atherosclerotic plaque composition determines the risk of plaque disruption.[2] In particular, high levels of tissue factor (TF), the main procoagulant protein, within the atherosclerotic lesion increase a plaque´s thrombogenicity.[3] Upon rupture of the plaque, the pathways that regulate platelet activation and fibrin (the final protein of the coagulation cascade) formation are intrinsically associated in the formation of a thrombus.[3, 4] In this regard, the COMPASS trial recently provided empirical evidence supporting the use of combined antiplatelet and anticoagulant therapy for reducing cardiovascular events and death in patients with atherosclerotic disease.[5, 6] These findings update the classical view, which recommends the use of antiplatelet in preference to anticoagulant therapy for secondary cardiovascular prevention.[3, 7, 8] With the new treatment paradigm, exploring new genes associated with antiplatelet and anticoagulant properties, with minor impact on bleeding risk, is considered to be a priority in the cardiovascular field.[3, 9, 10]

Apolipoprotein L domain-containing protein 1 (*Apold1*), also known as vascular early response gene, is a gene identified in endothelial cells[11] that responds to different stimuli such as ischemia, cytokines, growth factors and stress.[12, 13] Genes selectively expressed in endothelial cells could primarily determine thrombus formation.[10, 14] However, whether *Apold1* directly contributes to thrombosis has not yet been investigated. Therefore, the purpose of this study was to assess the effect of *Apold1* on arterial thrombus formation using an *in vivo* model of carotid thrombosis induced by photochemical injury.

Material and methods

Animals

Experiments were performed with 11–15-week-old male *Apold1* knockout (*Apold1*$^{-/-}$) mice and wild-type (WT) control C57Bl6/J littermates. In the Apold1(-/-) mice, the open reading frame was replaced with a beta-gal/neo cassette.[15] These mice were generously provided by Dr. Paul Worley and backcrossed to C57Bl6/J mice for more than 6 generations. Genotype confirmation was performed by PCR using the following primers:F2 5′-CTCTAGCCTAGGGCAGCAAC-3′; wtR1 5′-GAGAGAGGTCGGACGTGATG-3′; LacZR 5′-GGCGATTAAGTTGGGTAACG-3′ (Regard and Worley, unpublished data). PCR cycling was performed at 95°C for 5 min followed by 45 cycles at 95°C for 30 s, 64°C for 30 s, 72°C for 30 s and finally 72°C for 5 min, by using Taq DNA polymerase (Sigma-Aldrich, D1806-1.5KU) supplemented with 1M Betaine (Sigma-Aldrich, B0300-1VL). PCR products were analyzed by 2% agarose gel electrophoresis. Study design and experimental protocols were approved by the institutional animal care committee (License no. TVA 153_2018 Kommission für Tierversuche des Kantons Zürich, Switzerland).

Laser-induced carotid thrombosis *in vivo*

In vivo mouse carotid thrombosis was induced as previously described.[16-18] Briefly, animals were anaesthetized using 87 mg/kg sodium pentobarbital (Butler, Columbus, OH, USA). The right common carotid artery was exposed following a midline cervical incision. A Doppler flow probe (Transonic Systems, Ithaca, NY, USA) connected to a flowmeter (Model T106, Transonic Systems, Ithaca, NY, USA) was used for continuous assessment of carotid blood flow and heart rate. To induce photochemical injury of the endothelium, Rose Bengal (63 mg/kg body weight) was injected into the tail vein and the right common carotid artery was exposed to a laser light beam (1.5 mW, 540 nm, Mellesgriot Inc., Carlsbad, CA, USA) at a distance of 6 cm for 60 min. Blood flow was monitored from laser onset for maximum 120 min, or until occlusion (flow ≤0.1 mL for 1 min) occurred. During the experimental period, the operator was blinded to the animal group allocation. After thrombosis,

animals were euthanized by inhalation of an isoflurane overdose to allow the harvesting of blood and carotid arteries.

Tissue factor activity assay

TF activity was determined as previously described[19, 20] in murine plasma and left carotid arteries from *Apold1*$^{-/-}$ and WT mice by colorimetric assay, according to the recommendation of the manufacturer (American Diagnostica, Stamford, CT, USA). Blood was collected via intracardiac puncture and immediately mixed with EDTA, the EDTA-blood solution was then centrifuged for 15 min at 3000 g. EDTA-plasma was collected and snap frozen in liquid nitrogen. Carotid arteries were lysed (50mmol/L Tris–HCl, 100mmol/L NaCl, 0.1% Triton X-100, pH 7.4), and total protein concentration was determined by Bradford protein assay according to the manufacturer's recommendations (VWR Life Science AMRESCO, Solon, OH, USA). EDTA-plasma and carotid lysates were mixed with factor VIIa and X which leads to the conversion of factor X to Xa; factor Xa subsequently cleaves the chromogenic substrate SPECTROZYME FXa. Optical density of cleaved SPECTROZYMEV FXa was determined at 490 nm by Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and subtracted from absorbance at 405 nm. Finally, TF (pM) content was calculated, according to a standard curve. For carotid lysates, TF concentration as detected by the colorimetric assay was normalized to the total protein content of the sample and expressed as pM/g of total protein.

Collagen-induced platelet aggregation in mice

For platelet aggregation studies, washed platelets were obtained from 3.8 % citrate anticoagulated blood after euthanasia, as previously described[21].Thereafter, washed platelets were re-suspended in Thyrode's buffer (134 mM NaCl; 0.34 mM Na2HPO4; 2.9 mM KCl; 12 mM NaHCO3; 20 mM Hepes; 5 mM glucose; 0.35% (w/v) bovine serum albumin; pH 7.0) and platelet counts were normalized to 200'000/µl. Platelets were activated with collagen (final concentration 5 µg/ml) and maximal aggregation (%), lag phase (s) and slope of aggregation (%/min) were assessed using light

transmission aggregometry (APACT 4004 aggregometer, Haemochrom Diagnostica GmbH, Essen, Germany).

Blood cell counts

Total blood cell count was performed on a ScilVet ABCplus (Horiba, Kyoto, Japan) using EDTA-anticoagulated blood.

Western Blotting

Protein expression was determined by Western blot analysis as previously described.[22, 23] Aortae were homogenized in lysis buffer (Tris 50 mM, NaCl 150 mM, EDTA 1 mM, NaF 1 mM, DTT 1 mM, aprotinin 10 mg/mL, leupeptin 10 mg/mL, Na3VO4 0.1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, and NP-40 0.5%). Protein concentration was determined according to the manufacturer's recommendations (VWR Life Science AMRESCO, Solon, OH, USA); 20 – 30 µg of total protein lysates were separated on an 8 or 10% SDS–PAGE before being transferred to a polyvinylidene fluoride membrane by wet transfer (Bio-Rad). Membranes were incubated with primary antibodies against phospho-Akt (1:1000, Cell Signaling, Beverly, MA, USA), Akt (1:1000, Cell Signaling, Beverly, MA, USA), phospho-p38 (1:500, Cell Signaling, Beverly, MA, USA), p38 (1:1000, Cell Signaling, Beverly, MA, USA), phospho- c-Jun N-terminal kinase (JNK) (1:1000, Cell Signaling, Beverly, MA, USA), JNK (1:1000, Cell Signaling, Beverly, MA, USA), phospho-extracellular-signal-regulated kinase (ERK) (1:1000, Cell Signaling, Beverly, MA, USA), ERK (1:1000, Cell Signaling, Beverly, MA, USA) at 4°C overnight on a shaker. Secondary antibodies (Southern Biotechnology, Birmingham, AL, USA) were applied for 1 h at room temperature. Densitometric analyses were performed (Amersham Imager 600, GE Healthcare Europe GmbH, Glattbrugg, Switzerland) and protein expression of the phosphorylated protein was normalized to the total one.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from aortae using TRI reagent (Sigma-Aldrich, Buchs, Switzerland), according to the recommendations of the manufacturer. Conversion of the total cellular RNA to cDNA was performed with Moloneymurine leukaemia virus reverse transcriptase and random hexamers (GE

Healthcare, Chicago, IL, USA) in a final volume of 35 µl, using 2 µg of total RNA, according to recommendations of the manufacturer. RT-PCR was performed in a QuantStudio 7 Flex RT-PCR cycler (Applied BiosystemsTM, Foster City, CA), according to the instructions of the manufacturer. All RT- PCR experiments were performed using the SYBR Select Master Mix provided by Applied Biosystems (Life Technologies, Carlsbad, CA, USA). Each reaction (20 µl) contained 2 µl cDNA, 1µl of each primer and 10 µl of Master Mix. The amplification program consisted of 1 cycle at 95ºC for 10 min, followed by 40 cycles with a denaturing phase at 95ºC for 15s and an annealing/elongation phase at 60ºC for 1 min. Cycle threshold (Ct) values for each gene were obtained for each sample and differences in Ct values between a test gene and endogenous controls were calculated and used for statistical analyses. For quantitative RT-PCR, the following primers were used; for mouse TF: sense primer (1) 50-CTTTCTCATGTCCAGGGT-30, antisense primer (1) 50-GTTGCCAAACCTCTTTGT-30 and GAPDH: (1) 50-CTTTCTCATGTCCAGGGT-30, antisense primer (1) 50-GTTGCCAAACCTCTTTGT-30.

Results

Deficiency of *Apold-1* accelerates time to arterial thrombotic occlusion

To evaluate the role of *Apold1* in arterial thrombosis, *Apold1*$^{-/-}$ and WT mice underwent endothelial injury by generating reactive oxygen species using a photoreactive dye (i.e. Rose Bengal). Body weight (WT: 28.91 ± 0.66 g *vs.* *Apold1*$^{-/-}$: 29.19 ± 0.56 g; *P* >0.05; n=12-14, **Figure 1A**), initial heart rate (WT: 264.3 ± 8.80 bpm/min *vs. Apold1*$^{-/-}$: 289.2 ± 12.63 bpm/min; *P* >0.05; n=12-13, **Figure 1B**) and initial carotid blood flow (WT: 0.59 ± 0.036 ml/min *vs. Apold1*$^{-/-}$: 0.59 ± 0.029 ml/min; *P* >0.05; n=12-14, **Figure 1C**) did not differ between groups. WT mice developed carotid artery thrombosis within a mean time of 39.96 ± 3.86 min, while *Apold1*$^{-/-}$ mice occluded within a mean time of 29.42 ± 2.74 min (P < 0.05; n=12-14, **Figure 2D,E**).

Deficiency of *Apold-1* increases TF activity and decreases phosphatidyl-inositol-3 kinase (PI3K)/Akt activation, but does not affect TF mRNA levels and mitogen-activated protein kinases (MAPKs) activation

To investigate the mechanisms underlying the prothrombotic phenotype observed in *Apold1-/-* mice, TF expression and activity was assessed in *Apold1*$^{-/-}$ and WT mice. Deficiency of *Apold1* did not affect TF gene expression (WT: 0.04 ± 0.016 ΔCt vs. *Apold1*$^{-/-}$: 0.02 ± 0.003 ΔCt; *P* > 0.05; n=6-8, **Figure 2A**), but increased TF activity in the carotid artery (WT:13.48 ± 3.20 pM/g vs. *Apold1*$^{-/-}$: 27.31 ± 4.55 pM/g; *P* <0.05; n=10, **Figure 2B**). In addition plasma levels of TF did not differ between groups (WT:183.9 ± 45.92 pM *vs. Apold1*$^{-/-}$: 308.0 ± 59.33 pM; *P* >0.05; n=9-10, **Figure 2C**).To assess the potential mechanisms explaining the higher TF activity in *Apold1*$^{-/-}$ mice, we evaluated the signaling pathways regulating TF. Activation of MAPK, p38 (WT:1.41 ± 0.169 AU vs. *Apold1*$^{-/-}$: 1.32 ± 0.178 AU; *P* > 0.05; n=8-9, **Figure 2D**), JNK (WT:1.00 ± 0.236 AU vs. *Apold1*$^{-/-}$: 0.80 ± 0.101 AU; *P* > 0.05; n=7-9, **Figure 2E**), and ERK (WT:0.397 ± 0.184 AU vs. *Apold1*$^{-/-}$: 0.196 ± 0.057 AU; *P* > 0.05; n=6-7, **Figure 2F**), which regulate TF at transcriptional level, were unaffected by deletion of *Apold1*. Further, we evaluated the activation status of the PI3K/Akt pathway, a negative regulator of TF which functions at both the transcriptional or post-transcriptional level. Importantly, the activation

of Akt, assessed as the ratio between phosphorylated Akt and total Akt, was decreased in the arteries of *Apold1*$^{-/-}$ mice as compared to WT mice (WT:1.46 ± 0.289 AU vs. *Apold1*$^{-/-}$: 0.77± 0.143 AU; P <0.05; n=6-8, **Figure 2G**).

Deficiency of *Apold-1* increases platelet reactivity to collagen

Given the importat role played by platelets in arterial thombus formation, we investigated whether *Apold-1* deficiency affects platelet count and function. Complete blood count analyses revealed that platelet number (WT:766 ± 20 10^3/mm^3 vs. *Apold1*$^{-/-}$: 814 ± 61.63 10^3/mm^3 ; P > 0.05; n=9-10, **Figure 3A**) and volume (WT:7.03 ± 0.33 fl vs. *Apold1*$^{-/-}$: 6.77 ± 0.32 fl; P > 0.05; n=9-10, **Figure 3B**) were unchanged in Apold1$^{-/-}$ mice. In addition, reactivity of washed platelets to collagen was assessed by light transmission aggregometry. Deficiency of *Apold-1* enhanced collagen-induced platelet reactivity as demonstrated by increased maximal aggregation (WT:40.46 ± 5.93 % vs. *Apold1*$^{-/-}$: 68.49 ± 6.88 %; P < 0.05; n=9-10, **Figure 3C**), increased rate (slope) of aggregation (WT:16.79 ± 3.5 %/min vs. *Apold1*$^{-/-}$: 47.13 ± 12.35; P < 0.05; n=9-10, **Figure 3D**) and decreased lag phase (WT:175.5 ± 24.05 s vs. *Apold1*$^{-/-}$: 106.6 ± 21.73 s; P <0.05; n=9-10, **Figure 3E**).

Discussion

This study assessed the role of a gene specifically expressed in endothelial cells, *Apold1*, in arterial thrombosis using an *in vivo* mouse model of arterial thrombus formation relying on a laser induced endothelial-specific vascular injury. The main findings are: 1) *Apold1* deficiency leads to increased arterial thrombus formation in the carotid artery, 2) arterial TF activity is increased while PI3K activation is decreased and 3) platelet reactivity is increased in response to collagen. These results denote *Apold1* as a novel gene regulating arterial thrombosis by acting on the coagulation cascade and platelet activation.

Little is known about the function of *Apold1* in health and disease, however it appears to be involved in several biological processes. Loss of *Apold1* reduces oedema formation in a mouse model of stroke,[15] and angiogenesis and neurogenesis in long-term recovery from neonatal stroke.[11] Additionally, intense physical activity as well as stress exposure strongly activate Apold1 expression in the heart and in the brain, [24,25] and the stress-induced increase is mediated by beta2-adrenergic receptors.[13] The regulation by adrenoceptors might explain why various physiological stimuli can trigger Apold1 expression. Despite its widespread involvement in different biological functions, *Apold1* deficient mice are healthy and appear to have no overt behavioral alterations.[11, 15] Accordingly, *Apold1* may not be essential under physiological conditions, however, whether *Apold1* plays crucial functions under pathological conditions deserves further investigation.

In the present study, our results demonstrated a protective role of *Apold1* in arterial thrombosis. TF activates the extrinsic coagulation cascade following the disruption of vascular integrity.[26,27] In line with its physiological function,[28] TF is normally expressed by subendothelial cells (i.e. adventitia and to lesser extent in medial cells of the arterial wall), which are not in direct contact with circulating coagulation factors. However, a growing number of evidence suggests that TF may also be induced by inflammatory molecules or be present in an inactive form (encrypted) in endothelial and circulating cells.[14, 28, 29] In this respect, the present study found that TF within the arterial wall was augmented in *Apold1*$^{-/-}$ mice. This result indicates that increased vascular TF activity may be a primary contributor to

arterial thrombosis, thereby supporting previous research implying a greater contribution of vessel wall versus hematopoietic-produced TF to thrombus formation.[30]

The lack of a cell-type specific knockout model limits our ability to ascertain which cell type is responsible for the prothrombotic phenotype observed in *Apold1*$^{-/-}$ mice. However, considering that the endothelium is the only vascular cell in which *Apold-1* is expressed,[12] it is reasonable to infer a predominant endothelium-dependent increase in vascular TF activity. In this regard, several reports have described a major contribution of endothelium-derived TF to arterial thrombosis.[4, 16, 29] Endothelial TF can be regulated at transcriptional as well as posttranscriptional levels.[31] In this study, TF mRNA levels were not different between groups, consistent with our finding of unaltered MAPKs activation in *Apold1*$^{-/-}$ mice. Conversely, the PI3K/Akt pathway, a negative regulator of endothelial TF at both transcriptional and posttranscriptional levels, was decreased in *Apold1*$^{-/-}$ mice; thus, the decreased activation of the PI3K/Akt pathway could at least in part explain the increased TF activity observed in *Apold1*$^{-/-}$ mice. Nevertheless, we cannot rule out an alteration in the activation status (encryption/decryption) of TF, as observed in other studies.[29, 32] Notwithstanding, this hypothesis is difficult to test due to the lack of knowledge on the underlying mechanisms of this process.

Another important result of this study is that *Apold1*$^{-/-}$ mice display an isolated platelet phenotype which, in concert to the augmented vascular TF activity, provides a possible explanation for the increased thrombogenicity observed in *Apold1*$^{-/-}$ mice. Our results indicate that *Apold1*$^{-/-}$ deficiency increases platelet reactivity in response to the physiological agonist collagen, which plays a key role in the initial platelet response in atherothrombotic events. Platelets specifically and firmly bind to collagen via their glycoprotein VI (GPVI) receptor, which undergoes activation upon ligand binding. Thereafter, platelet degranulation with ensuing autocrine and paracrine stimulation, P-selectin exposure, and conformational activation of the platelet fibrinogen receptor GPIIb/IIIa mediate formation of the platelet thrombus.

Thus, the increased sensitivity to collagen may be explained by alterations in the number or affinity of platelet GPVI or by alterations in platelet-signalling, which could influence platelet degranulation, platelet thromboxane 2 generation and GPIIb/IIIa receptor activation.[33] Thus, further research will be

needed to elucidate the mechanisms by which *Apold1* regulates platelet function, starting with assessing platelet reactivity in response to other agonists, such as thrombin and ADP. However, in the context of atherothrombosis with exposure of subendothelial collagen as a key pathophysiological feature, increased platelet sensitivity to collagen is of utmost importance. Noteworthy, these experiments were performed on washed platelets, thereby excluding plasmatic or vascular influences on platelet activation – most importantly, platelet activation via the TF-thrombin pathway. Thus, platelets may play a key role in the prothrombotic phenotype observed in *Apold1*$^{-/-}$ mice. Additional experiments using aspirin and FXa inhibitor may be an elegant way to determine the contribution of the observed platelet and coagulation phenotype to thrombus formation.

In summary, this study demonstrates that *Apold1* deletion increases coagulation and thrombus formation. Specifically, Apold1 deficiency increased vascular TF activity and enhanced platelet reactivity to collagen in a mouse model of acute carotid artery thrombosis. Findings of the present study call for further investigation to assess the potential role of *Apold1* as a novel therapeutic target in the settings of acute arterial thrombosis.

Acknowledgments

We thank Martin Roszkowski and Han-Yu Lin for genotyping of *Apold1*$^{-/-}$ mice, Paul Worley for generously sharing the initial breeding pairs of *Apold1*$^{-/-}$ mice, and Jean Reagard for helpful comments and discussions regarding *Apold1* function.

The present work was supported by the Swiss Heart Foundation, the Swiss National Science Foundation [310030_175546], the Alfred and Annemarie von Sick Grants for Translational and Clinical Research Cardiology and Oncology, and the Foundation for Cardiovascular Research–Zurich Heart House to G.G. C. In addition, G.G.C. is the recipient of a Sheikh Khalifa's Foundation Assistant Professorship at the Faculty of Medicine, University of Zurich. JB is funded by the ETH Zurich, SNSF Project Grant 310030_172889/1, the Forschungskredit of the University of Zurich (grant no. FK-15-035), and the Novartis Foundation for Medical Biological Research. C. D.-C. is the recipient of the Early Postdoc Mobility Grant from the Swiss National Foundation (SNF).

Authors' contributions

CD and NB participated in the acquisition of data, analysis, interpretation, prepared figures and wrote the manuscript; PW and VN participated in the acquisition of data; JB and GC designed the experiments; LL, JHB, FM,TFL, JB, GC revised the manuscript critically for important intellectual contents. All authors read and approved the final manuscript.

Disclosures

The authors declare no competing financial interests exist.

Figures Legend

Figure 1: *Apold1* deficiency accelerates time to carotid thrombotic occlusion in vivo. (**A, B , C**) Body weight, blood flow and heart rate in WT and *Apold1*$^{-/-}$ mice. (**D**) Representative mean blood flow recordings of WT and *Apold1*$^{-/-}$ mice after *in vivo* photochemical injury. Occlusion was defined as blood flow below 0.1 mL/min for at least 1 min. (**E**) Time to thrombotic occlusion in WT and *Apold1*$^{-/-}$ mice. Data are expressed mean ± SEM. **P* <0.05

Figure 2: Effect of *Apold1* deficiency on TF gene expression, TF activity, MAPKs and Akt signaling pathways. (**A**) TF mRNA expression in mouse aorta from *Apold1*$^{-/-}$ and WT mice. (**B**) TF activity in carotid arteries and **(C)** plasma of WT and *Apold1*$^{-/-}$ mice (**D, E, F,G**) Western blot of MAPKs (p38, JNK and ERK) and Akt pathway in mouse aorta from *Apold1*$^{-/-}$ and WT mice. Data are expressed mean ± SEM. **P* <0.05

Figure 3: *Apold1* deficiency enhances platelet reactivity to collagen. **(A,B)** Platelet counts and volumes in WT and *Apold1*$^{-/-}$ mice **(C)** Deficiency of *Apold 1* enhance collagen (5 µg/ml)-induced platelet aggregation demonstrated by increased maximal aggregation, **(D)** increased rate (slope) of aggregation **(E)** and decreased lag phase. Data are expressed mean ± SEM. **P* <0.05, ** *P* <0.01

Figure 1

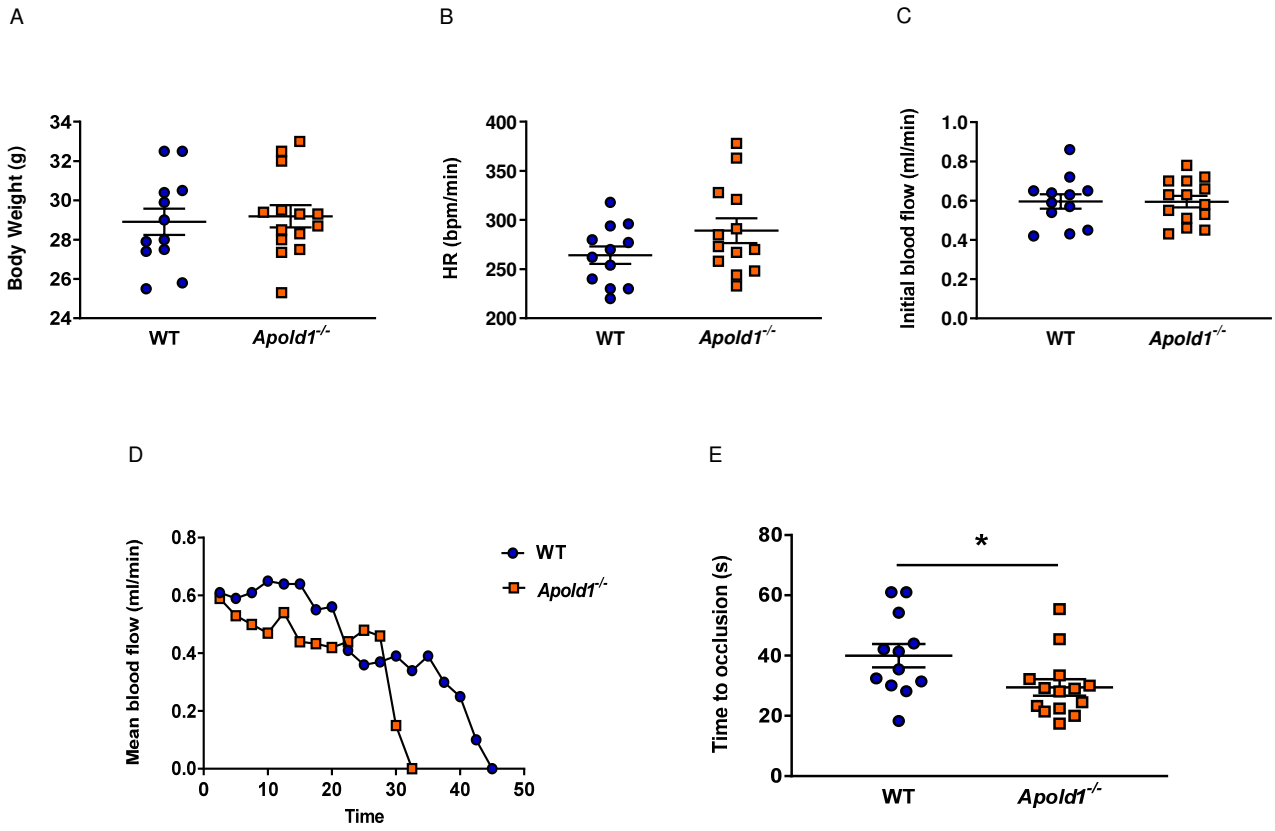

A
Body Weight (g)
34
32
30
28
26
24
WT
Apold1-/-
B
HR (bpm/min)
400
350
300
250
200
WT
Apold1-/-
C
Initial blood flow (ml/min)
1.0
0.8
0.6
0.4
0.2
0.0
WT
Apold1-/-
D
Mean blood flow (ml/min)
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Time
WT
Apold1-/-
E
Time to occlusion (s)
80
60
40
20
0
*
WT
Apold1-/-

Figure 2

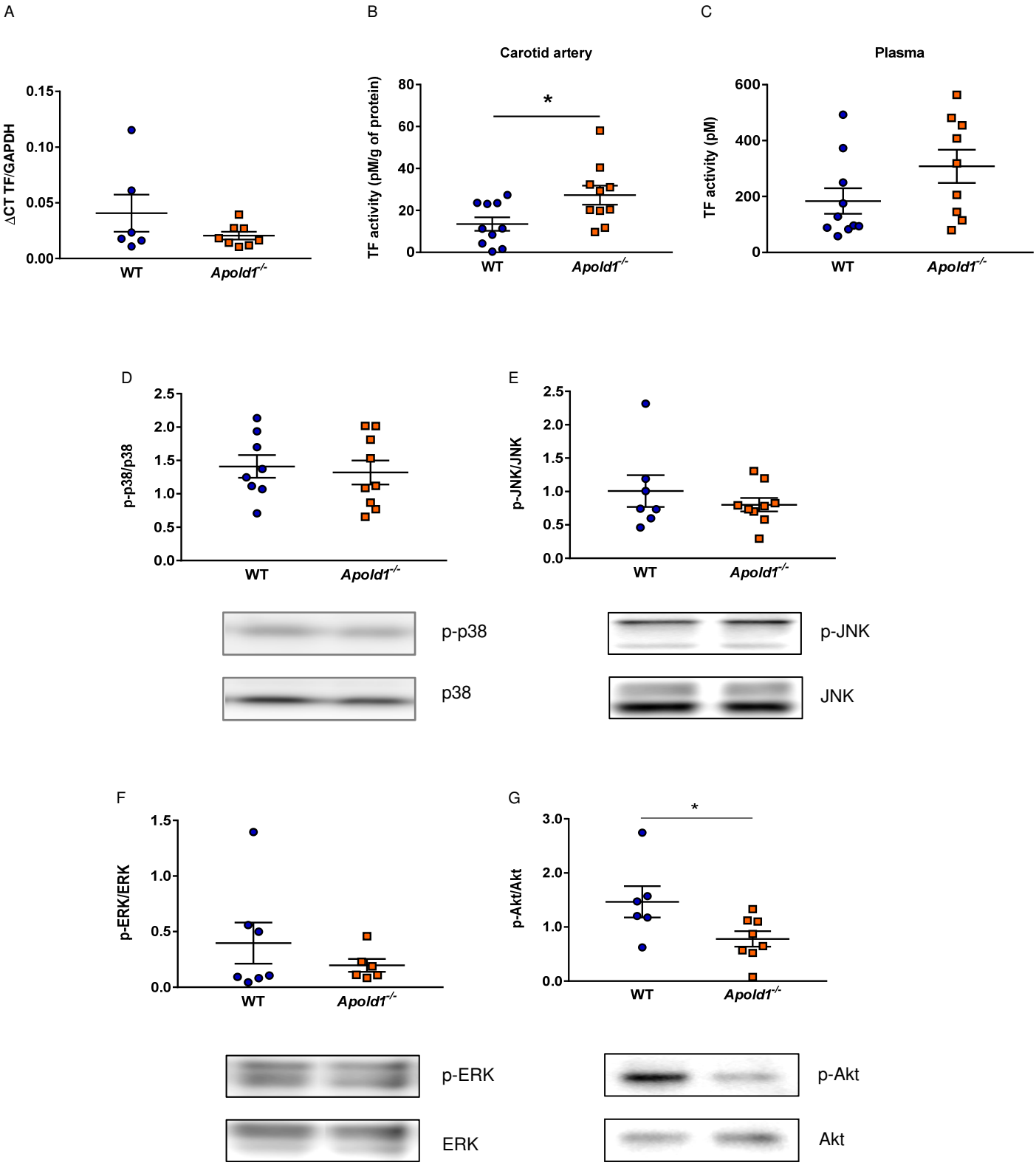
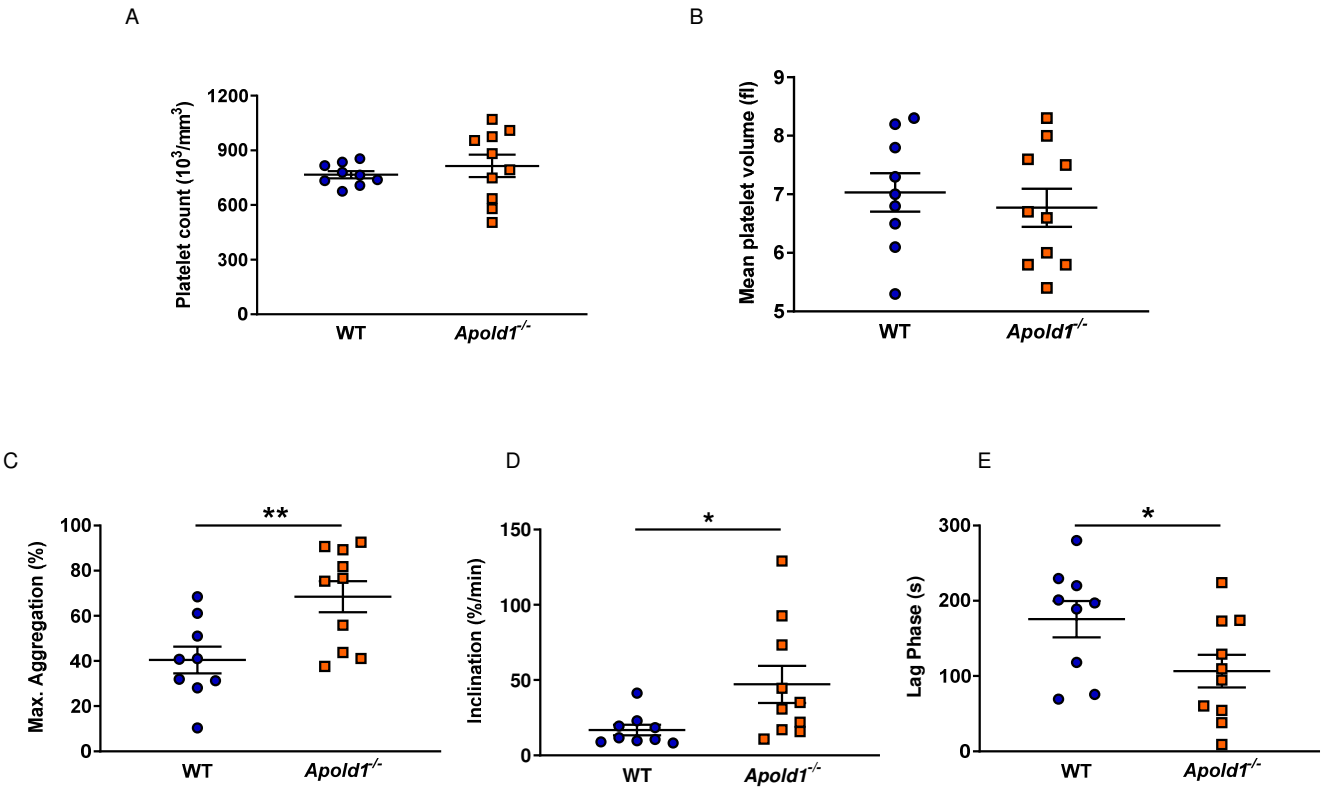

A
ΔCT TF/GAPDH
0.15
0.10
0.05
0.00
WT
Apold1-/-
B
Carotid artery
TF activity (pM/g of protein)
80
60
40
20
0
*
WT
Apold1-/-
C
Plasma
TF activity (pM)
600
400
200
0
WT
Apold1-/-
D
p-p38/p38
2.5
2.0
1.5
1.0
0.5
0.0
WT
Apold1-/-
p-p38
p38
E
p-JNK/JNK
2.5
2.0
1.5
1.0
0.5
0.0
WT
Apold1-/-
p-JNK
JNK
F
p-ERK/ERK
1.5
1.0
0.5
0.0
WT
Apold1-/-
p-ERK
ERK
G
p-Akt/Akt
3.0
2.0
1.0
0.0
*
WT
Apold1-/-
p-Akt
Akt

Figure 3


A
Platelet count (10³/mm³)
1200
900
600
300
0
WT
Apold1-/-
B
Mean platelet volume (fl)
9
8
7
6
5
WT
Apold1-/-
C
Max. Aggregation (%)
100
80
60
40
20
0
**
WT
Apold1-/-
D
Inclination (%/min)
150
100
50
0
*
WT
Apold1-/-
E
Lag Phase (s)
300
200
100
0
*
WT
Apold1-/-

References

1. Wendelboe AM and Raskob GE. Global Burden of Thrombosis: Epidemiologic Aspects. *Circ Res*. 2016;118:1340-7.

2. Viles-Gonzalez JF, Fuster V and Badimon JJ. Atherothrombosis: a widespread disease with unpredictable and life-threatening consequences. *Eur Heart J*. 2004;25:1197-207.

3. Olie RH, van der Meijden PEJ and Ten Cate H. The coagulation system in atherothrombosis: Implications for new therapeutic strategies. *Res Pract Thromb Haemost*. 2018;2:188-198.

4. Reiner MF, Akhmedov A, Stivala S, Keller S, Gaul DS, Bonetti NR, Savarese G, Glanzmann M, Zhu C, Ruf W, Yang Z, Matter CM, Luscher TF, Camici GG and Beer JH. Ticagrelor, but not clopidogrel, reduces arterial thrombosis via endothelial tissue factor suppression. *Cardiovasc Res*. 2017;113:61-69.

5. Eikelboom JW, Connolly SJ, Bosch J, Dagenais GR, Hart RG, Shestakovska O, Diaz R, Alings M, Lonn EM, Anand SS, Widimsky P, Hori M, Avezum A, Piegas LS, Branch KRH, Probstfield J, Bhatt DL, Zhu J, Liang Y, Maggioni AP, Lopez-Jaramillo P, O'Donnell M, Kakkar AK, Fox KAA, Parkhomenko AN, Ertl G, Stork S, Keltai M, Ryden L, Pogosova N, Dans AL, Lanas F, Commerford PJ, Torp-Pedersen C, Guzik TJ, Verhamme PB, Vinereanu D, Kim JH, Tonkin AM, Lewis BS, Felix C, Yusoff K, Steg PG, Metsarinne KP, Cook Bruns N, Misselwitz F, Chen E, Leong D, Yusuf S and Investigators C. Rivaroxaban with or without Aspirin in Stable Cardiovascular Disease. *N Engl J Med*. 2017;377:1319-1330.

6. Nicholls SJ and Nelson AJ. Rivaroxaban With or Without Aspirin for the Secondary Prevention of Cardiovascular Disease: Clinical Implications of the COMPASS Trial. *Am J Cardiovasc Drugs*. 2019.

7. Beer JH and Bonetti N. A COMPASS to REACH the right patients with thrombocardiology: benefits, risks, and future of the new concept. *Eur Heart J*. 2018;39:758-761.

8. Smith SC, Jr., Benjamin EJ, Bonow RO, Braun LT, Creager MA, Franklin BA, Gibbons RJ, Grundy SM, Hiratzka LF, Jones DW, Lloyd-Jones DM, Minissian M, Mosca L, Peterson ED, Sacco RL, Spertus J, Stein JH, Taubert KA, World Heart F and the Preventive Cardiovascular Nurses A. AHA/ACCF Secondary Prevention and Risk Reduction Therapy for Patients with Coronary and other

Atherosclerotic Vascular Disease: 2011 update: a guideline from the American Heart Association and American College of Cardiology Foundation. *Circulation*. 2011;124:2458-73.

9. Colman RW. Are hemostasis and thrombosis two sides of the same coin? *J Exp Med*. 2006;203:493-5.

10. Baaten C, Meacham S, de Witt SM, Feijge MAH, Adams DJ, Akkerman JN, Cosemans J, Grassi L, Jupe S, Kostadima M, Mattheij NJA, Prins MH, Ramirez-Solis R, Soehnlein O, Swieringa F, Weber C, White JK, Ouwehand WH and Heemskerk JWM. A synthesis approach of mouse studies to identify genes and proteins in arterial thrombosis and bleeding. *Blood*. 2018;132:e35-e46.

11. Mirza MA, Capozzi LA, Xu Y, McCullough LD and Liu F. Knockout of vascular early response gene worsens chronic stroke outcomes in neonatal mice. *Brain Res Bull*. 2013;98:111-21.

12. Regard JB, Scheek S, Borbiev T, Lanahan AA, Schneider A, Demetriades AM, Hiemisch H, Barnes CA, Verin AD and Worley PF. Verge: a novel vascular early response gene. *J Neurosci*. 2004;24:4092-103.

13. Roszkowski M, Manuella F, von Ziegler L, Duran-Pacheco G, Moreau JL, Mansuy IM and Bohacek J. Rapid stress-induced transcriptomic changes in the brain depend on beta-adrenergic signaling. *Neuropharmacology*. 2016;107:329-338.

14. Wang M, Hao H, Leeper NJ, Zhu L and Early Career C. Thrombotic Regulation From the Endothelial Cell Perspectives. *Arterioscler Thromb Vasc Biol*. 2018;38:e90-e95.

15. Liu F, Turtzo LC, Li J, Regard J, Worley P, Zeevi N and McCullough LD. Loss of vascular early response gene reduces edema formation after experimental stroke. *Exp Transl Stroke Med*. 2012;4:12.

16. Akhmedov A, Camici GG, Reiner MF, Bonetti NR, Costantino S, Holy EW, Spescha RD, Stivala S, Schaub Clerigue A, Speer T, Breitenstein A, Manz J, Lohmann C, Paneni F, Beer JH and Luscher TF. Endothelial LOX-1 activation differentially regulates arterial thrombus formation depending on oxLDL levels: role of the Oct-1/SIRT1 and ERK1/2 pathways. *Cardiovasc Res*. 2017;113:498-507.

17. Breitenstein A, Stampfli SF, Reiner MF, Shi Y, Keller S, Akhmedov A, Schaub Clerigue A, Spescha RD, Beer HJ, Luscher TF, Tanner FC and Camici GG. The MAP kinase JNK2 mediates cigarette smoke-induced arterial thrombosis. *Thromb Haemost*. 2017;117:83-89.

18. Holy EW, Akhmedov A, Speer T, Camici GG, Zewinger S, Bonetti N, Beer JH, Luscher TF and Tanner FC. Carbamylated Low-Density Lipoproteins Induce a Prothrombotic State Via LOX-1: Impact on Arterial Thrombus Formation In Vivo. *J Am Coll Cardiol*. 2016;68:1664-1676.

19. Reiner MF, Breitenstein A, Holy EW, Glanzmann M, Amstalden H, Stampfli SF, Bonetti NR, Falk V, Keller S, Savarese G, Benussi S, Maisano F, Luscher TF, Beer JH, Steffel J and Camici GG. Ticagrelor, but not clopidogrel active metabolite, displays antithrombotic properties in the left atrial endocardium. *Eur Heart J*. 2017;38:916-919.

20. Camici GG, Steffel J, Amanovic I, Breitenstein A, Baldinger J, Keller S, Luscher TF and Tanner FC. Rapamycin promotes arterial thrombosis in vivo: implications for everolimus and zotarolimus eluting stents. *Eur Heart J*. 2010;31:236-42.

21. Dowling MR, Josefsson EC, Henley KJ, Hodgkin PD and Kile BT. Platelet senescence is regulated by an internal timer, not damage inflicted by hits. *Blood*. 2010;116:1776-8.

22. Diaz-Canestro C, Merlini M, Bonetti NR, Liberale L, Wust P, Briand-Schumacher S, Klohs J, Costantino S, Miranda M, Schoedon-Geiser G, Kullak-Ublick GA, Akhmedov A, Paneni F, Beer JH, Luscher TF and Camici GG. Sirtuin 5 as a novel target to blunt blood-brain barrier damage induced by cerebral ischemia/reperfusion injury. *Int J Cardiol*. 2018;260:148-155.

23. Liberale L, Diaz-Canestro C, Bonetti NR, Paneni F, Akhmedov A, Beer JH, Montecucco F, Luscher TF and Camici GG. Post-ischaemic administration of the murine Canakinumab-surrogate antibody improves outcome in experimental stroke. *Eur Heart J*. 2018;39:3511-3517.

24. Simonsen ML, Alessio HM, White P, Newsom DL and Hagerman AE. Acute physical activity effects on cardiac gene expression. *Exp Physiol*. 2010;95:1071-80.

25. Floriou-Servou A, von Ziegler L, Stalder L, Sturman O, Privitera M, Rassi A, Cremonesi A, Thony B and Bohacek J. Distinct Proteomic, Transcriptomic, and Epigenetic Stress Responses in Dorsal and Ventral Hippocampus. *Biol Psychiatry*. 2018;84:531-541.

26. Osterud B and Bjorklid E. Sources of tissue factor. *Semin Thromb Hemost*. 2006;32:11-23.

27. Stampfli SF, Akhmedov A, Gebhard C, Lohmann C, Holy EW, Rozenberg I, Spescha R, Shi Y, Luscher TF, Tanner FC and Camici GG. Aging induces endothelial dysfunction while sparing arterial thrombosis. *Arterioscler Thromb Vasc Biol*. 2010;30:1960-7.

28. Breitenstein A, Camici GG and Tanner FC. Tissue factor: beyond coagulation in the cardiovascular system. *Clin Sci (Lond)*. 2009;118:159-72.

29. Ebert J, Wilgenbus P, Teiber JF, Jurk K, Schwierczek K, Dohrmann M, Xia N, Li H, Spiecker L, Ruf W and Horke S. Paraoxonase-2 regulates coagulation activation through endothelial tissue factor. *Blood*. 2018;131:2161-2172.

30. Day SM, Reeve JL, Pedersen B, Farris DM, Myers DD, Im M, Wakefield TW, Mackman N and Fay WP. Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. *Blood*. 2005;105:192-8.

31. Lopez-Pedrera C, Jardi M, Ingles-Esteve J, Munoz-Canoves P, Dorado G, Velasco F and Felez J. Characterization of tissue factor expression on the human endothelial cell line ECV304. *Am J Hematol*. 1997;56:71-8.

32. Chen VM and Hogg PJ. Encryption and decryption of tissue factor. *J Thromb Haemost*. 2013;11 Suppl 1:277-84.

33. Yun SH, Sim EH, Goh RY, Park JI and Han JY. Platelet Activation: The Mechanisms and Potential Biomarkers. *Biomed Res Int*. 2016;2016:9060143.

34. Regard JB. Cloning and characterization of verge: a vascular early response gene. 2002.